Amendments to the Drawings

Replacement Sheets for Figures 5-8 are submitted concurrently herewith.

REMARKS/ARGUMENTS

A. Status of the Claims

Claims 1-9 were pending at the time of the Action. Claims 1-3 and 6-9 are amended. Support for the claim amendments can be found throughout the specification and in the original claims. No claims have been canceled or added. Therefore, claims 1-9 are pending and presented herein for reconsideration.

B. Replacement Sheets for Figures 5-8

Figures 5-8 are objected to under 37 CFR 1.84(b). Replacement Sheets for Figures 5-8 are submitted herewith. Applicant respectfully notes that no amendments are made to these Figure 5-8. Applicant requests that the objections to Figures 5-8 be withdrawn.

C. The Claim Objections Are Overcome

Claims 1, 2, 6, 7, and 8 are amended to remove the informalities noted in the Action. The objections are therefore moot.

D. Written Description and Indefiniteness Rejections

1. "MsPRP2" and "Alfin1"

Claims 2-3 and 6-9 are rejected under 35 U.S.C. § 112, first and second paragraphs, for allegedly lacking written description and being indefinite for the recitation of the terms "MsPRP2" and "Alfin1." Applicant respectfully disagrees.

a. Written Description

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991). The MsPRP2 promoter is well known to be a specific sequence. As the

sequence is disclosed in FIG. 2 in the application, the specification provides sufficient written description of its structure. Similarly, Alfin1 is a well known transcription factor having a specific structure and sequence. *See* specification at paragraph [0004]; *see also* Winicov and Bastola, (1999), *Plant Physiol* 120, 473-80 (Exhibit 1). Therefore, the present specification describes the claimed invention in sufficient detail that one of ordinary skill in the art can reasonably conclude that Appellants had possession of the claimed invention at the time of filing. Withdrawal of the rejection is therefore respectfully requested.

b. Indefiniteness

A proper evaluation of the claims under the second paragraph of 35 U.S.C. § 112 requires that the claims be read in light of the specification as interpreted by one of ordinary skill in the art. *North Am. Vaccine, Inc. v. American Cyanamid Co.*, 7 F.3d 1571, 1579, 28 USPQ 2d 1333, 1339 (Fed. Cir. 1993); *In re Moore*, 439 F.2d 1232, 1235 (C.C.P.A. 1971). As explained above, the terms "MsPRP2" and "Alfin1" are designations that are well known by those skilled in the art. This is evidenced throughout the specification and is supported by a brief review of the art. *See* Specification at paragraph [0004].

Use of a well known term of art in the specification without detailed definitions thereof does not render claims utilizing that same language indefinite. *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1556-58, 220 USPQ 303, 315-16 (Fed. Cir. 1983). In view of the fact that a person of ordinary skill would understand these terms, withdrawal of the rejection is respectfully requested.

2. Claim 9

Claim 9 is rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for omitting essential steps. Claim 9 currently reads "[a] method of bioremediating a field, the method comprising planting the transgenic seeds of claim 8, wherein planting the

transgenic seeds bioremediates the field." The rejection is moot and withdrawal is respectfully requested.

E. The Anticipation Rejection is Overcome

Claims 1 and 2 are rejected under 35 U.S.C. § 102(b) as being allegedly anticipated by Winicov (WO 99/53016). In particular, it is asserted that Winicov teaches an expression cassette capable of directing heterologous protein expression in plant roots comprising an MsPRP2 promoter and a heterologous gene operably linked to the MsPRP2 promoter.

Applicant respectfully traverses. However, in order to advance prosecution, the claims now require the presence of the MsPRP2 secretion signal. As claim is anticipated only if each and every element as set forth in the claim is found in a single prior art reference, Winicov fails to anticipate the current claims. MPEP § 2131. In particular, Winicov fails to teach a secretion signal, as the sequence 1-60 alone will not work as a **secretory** sequence and would be insufficient even as **a signal** sequence. Specifically, the +1 to +60 would not be effective for secretion, as the additional base pairs (+61 to 75) encode additional amino acids required for the structure of the functional signal sequence and its ultimate cleavage at the cell wall. After the protein is made in the cytoplasm and recognized as a cell wall protein by its signal sequence, it is delivered to the cell membrane/cell wall for insertion with cleavage of the signal sequence. The actual cleavage occurs between amino acids Alanine (encoded by nucleotides 70,71,72) and Cysteine (encoded by nucleotides 73,74,75). *See* Von, Heijne, G. (1983) *Eur. J. Biochem*, 133:17-21 (Exhibit 2). Thus the original designation for MsPRP2 proposed by Deutch and Winicov (1995) was a gene encoding a chimeric cell wall protein with an amino terminal signal sequence. A secretion sequence was never disclosed or suggested.

Furthermore, secretion was not expected as the endogenous alfalfa MsPRP2 protein is a cell wall protein. Subsequent identification of cell wall proteins in other species have shown their genes to have similar signal sequences with sequences strongly conserved in the 61-75 region. Therefore, it was expected that the 1-75 sequence would act as a signal for targeting the signal-reporter gene construct that produced proteins to the cell wall. Thus, it was expected that all the jellyfish green fluorescent protein (GFP) would end up in the cell wall and **not** be secreted to the outside. The finding that such constructs with the 1-75 sequence secreted GFP outside was therefore unpredicted. This complete (+1 to +75) sequence is included in Fig. 3, which contains the sequence of the promoter construct, ribosome binding site, complete secretory sequence and the GFP reporter gene. The secretory sequence is first disclosed in the present application, as previous work had disclosed only a signal sequence presumably targeting the MsPRP2 protein **only** to the cell wall.

Because Winicov fails to teach an expression cassette comprising the MsPRP2 secretion signal, the rejection fails. Applicant respectfully requests reconsideration and withdrawal of the rejection.

F. The Obviousness Rejections are Overcome

1. Winicov

Claims 3-6 are rejected under 35 U.S.C. § 103(a) as being obvious over Winicov. The Action asserts that it would be obvious to one of skill in the art make an expression cassette comprising the MsPRP2 promoter and a gene for a heterologous protein, and another promoter and an Alfin1 coding sequence. It is further asserted that it would have been obvious to transfect plants and plant cells with this expression cassette to produce a recombinant protein in such plant cells. Applicant respectfully traverses.

The failure of an asserted combination to teach or suggest each and every feature of a claim remains fatal to an obviousness rejection under 35 U.S.C. § 103. *See In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974) (emphasis added) (to establish *prima facie* obviousness of a claimed invention, all the claim features must be taught or suggested by the prior art); MPEP § 2143.03. Here, no *prima facie* case of obviousness has been established, as Winicov fails to teach or suggest an expression cassette comprising a MsPRP2 secretion signal.

As explained above, the current claims are directed to expression cassettes that require the presence of the MsPRP2 secretion signal together with a heterologous gene, causing the gene product to be secreted into the medium. In contrast, Winicov teaches only the cell wall protein, and as such teaches that Alfin1 would be retained inside the cell nucleus. Further, as discussed above, secretion was not expected as the endogenous alfalfa MsPRP2 protein is a cell wall protein.

The currently claimed invention demonstrates the efficient combination of two root specific components for heterologous gene expression in one transformation. For example, claim 3 encompasses an expression cassette comprising 1) the promoter fragment of MsPRP2 **plus** the MsPRP2 secretion signal directing expression of a heterologous gene, and 2) overexpression of Alfin1 from a different promoter, which will help to drive the MsPRP2 promoter under normal conditions, as Alfin1 is normally limiting in roots and cells.

Secretion of recombinant proteins is not disclosed or suggested by Winicov, as there was no teaching or suggestion of secreting recombinant proteins from the roots, and as such a skilled person would not look to Winicov for an expression system which would allow secretion from the roots. Furthermore, using the promoter and signal sequence of MsPRP would not have been obvious to one of skill in the art, as Winicov does not disclose or suggest that such a secretion sequence is present in MsPRP2. Finally, the specific sequence for secretion used in the present

application would not be obvious from Winicov as there is no disclosure of the MsPRP2 sequence from +1 to +75.

In light of the currently pending claims, the cited references do not describe or suggest all elements of the invention, and therefore the Action has not established a *prima facie* case of obviousness. If the examiner does not produce a *prima facie* case, the applicant is under no obligation to submit evidence of nonobviousness." MPEP § 2142. Because Winicov fails to teach an expression cassette comprising the MsPRP2 secretion signal, the rejection fails as the reference fails to teach or suggest an element of each claim. Applicant respectfully requests reconsideration and withdrawal of the rejection.

2. Winicov in view of Lee

Claim 7 is rejected under 35 U.S.C. § 103(a) as being obvious over Winicov in view of Lee *et al.* (U.S. 6,020,169). Lee is cited as teaching the production of secreted proteins in plant cells with an expression cassette comprising a promoter, a sequence encoding a secretion signal peptide, and a protein coding sequence. Based on this and the teachings of Winicov discussed above, it is asserted that the currently claimed subject matter would have been obvious to one of skill in the art. Applicant respectfully traverses.

As explained above, Winicov fails to teach an expression cassette comprising the MsPRP2 secretion signal in combination with the MsPRP2 promoter or fragment thereof, and a protein. Lee fails to remedy this omission. In particular, Lee also fails to teach or suggest the MsPRP2 secretion signal sequence. As such, the Action fails to establish that the cited combination teaches or suggests every feature of the current claims, therefore failing to establish a *prima facie* case of obviousness.

Furthermore, there is no apparent reason to combine the cited references in the fashion claimed under a *KSR* analysis. *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398 (2007). In

particular, one of skill in the art would not look to Winicov for solutions relating to secretion of a protein, as Winicov does not teach the incorporation of a secretion signal. Furthermore, neither reference teaches or suggests the presence of a secretion signal in MsPRP2, and as such one of skill in the art would have no reason to attempt to use a secretion signal from MsPRP2. It appears that the Examiner is relying on hindsight to find a motivation to combine these references. The use of hindsight, however, is not appropriate to establish a motivation to combine. *See W.L. Gore Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir. 1983); MPEP § 2143.01.

For the reasons set forth above, claim 7 is not obvious. Reconsideration and withdrawal of the obviousness rejection is respectfully requested.

3. Winicov in view of Scheres

Claims 8-9 are rejected under 35 U.S.C. § 103(a) as being obvious over Winicov in view of Scheres *et al.* (U.S. Pub. 2004/0067506). Scheres is cited as teaching the use of transgenic plants for bioremediation. Based on this and the teachings of Winicov discussed above, it is asserted that the currently claimed subject matter would have been obvious to one of skill in the art. Applicant respectfully traverses.

As explained above, Winicov fails to teach the use of a secretion signal sequence in combination with the MsPRP2 promoter sequence or fragment thereof, and a protein. Scheres fails to remedy this omission. In particular, neither Winicov or Scheres disclose or suggest the existence or use of the MsPRP2 secretion signal. Thus, the combination of Winicov and Scheres does not describe all elements of the current claims. As such, the Action fails establish a *prima facie* case of obviousness.

Furthermore, there is no apparent reason to combine the cited references in the fashion claimed under a *KSR* analysis. *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398 (2007). In

particular, one of skill in the art would not look to Winicov for solutions relating to secretion of a protein, as Winicov does not teach the incorporation of a secretion signal. Furthermore, neither reference teaches or suggests the presence of a secretion signal in MsPRP2, and as such one of skill in the art would have no reason to attempt to use a secretion signal from MsPRP2.

In light of the above, the claims are not obvious over Winicov in view of Scheres. Applicant respectfully requests the withdrawal of the rejection.

G. Conclusion

In light of the foregoing, applicant respectfully submits that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. The examiner is invited to contact the undersigned attorney with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

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Transgenic Overexpression of the Transcription Factor *Alfin1* Enhances Expression of the Endogenous *MsPRP2* Gene in Alfalfa and Improves Salinity Tolerance of the Plants[1]

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Alfin1 **cDNA encodes a putative transcription factor associated with NaCl tolerance in alfalfa (*Medicago sativa* L.). The recombinant protein binds DNA in a sequence-specific manner, including promoter fragments of the NaCl-inducible gene *MsPRP2*. Alfin1 function was tested in transgenic alfalfa under the control of the 35S promoter in the sense and antisense orientations with the endogenous *MsPRP2* as a reporter gene. Calli overexpressing *Alfin1* were more resistant to growth inhibition by 171 mM NaCl than vector-transformed controls, whereas calli expressing *Alfin1* in the antisense orientation were more sensitive to NaCl inhibition. Transgenic plants overexpressing *Alfin1* in the sense orientation grew well. In contrast, the antisense transgenic plants grew poorly in soil, demonstrating that *Alfin1* expression is essential for normal plant development. Transgenic calli and plant roots overexpressing *Alfin1* showed enhanced levels of endogenous *MsPRP2* mRNA accumulation. However, *MsPRP2* mRNA accumulation was also regulated in a tissue-specific manner, as shown in leaves of transgenic plants overexpressing *Alfin1*. These results suggest that Alfin1 acts as a transcriptional regulator in plants and regulates *MsPRP2* expression in alfalfa. *Alfin1* overexpressing transgenic plants showed salinity tolerance comparable to one of our NaCl-tolerant plants, indicating that *Alfin1* also functions in gene regulation in NaCl tolerance.**

Plants and cells adapt to changes in the ionic environment as a result of salinity and drought through temporal or sustained regulation of a large number of genes (for review, see Bohnert et al., 1995; Ingram and Bartels, 1996; Bray, 1997), but the molecular mechanisms responsible for this regulation have remained elusive. We have documented coordinated gene regulation in long-term acquired NaCl tolerance in alfalfa (*Medicago sativa* L.) and rice (Winicov et al., 1989; Winicov, 1991, 1996) and have been interested in defining a functional role for a putative transcription factor, Alfin1, in the altered gene expression in NaCl-tolerant alfalfa (Winicov, 1993; Bastola et al., 1998).

A relatively small number of transcription factors have been identified to date that bind to promoter elements in genes regulated by NaCl/drought stress (for review, see Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Winicov and Bastola, 1997), and much of the information has been gene specific. A more complex view of transcriptional regulation is implied by the requirement of a coupling element for stress regulation of the barley *HVA22* gene containing the ABA response element (Shen et al., 1996) and the combined role of *myc* and *myb* transcriptional activators in ABA- and dehydration-inducible expression of a promoter region of the *rd22* gene (Abe et al., 1997). The potential interactions of various factors is compounded further in that transcription factors such as myc and myb belong to extensive multigene families with tissue-specific expression patterns. Nevertheless, recent reports have shown that ectopic expression of transcriptional activators can result in changes in plant responses to cold (Jaglo-Ottosen et al., 1998) and disease resistance (Cao et al., 1998) and changes in metabolic products in plants (Tamagnone et al., 1998) and cultured cells (Grotewold et al., 1998) by affecting the levels of expression of endogenous genes, indicating the possibility of testing the function of individual transcription factors.

Alfin1 cDNA encodes a novel member of the zinc-finger family of proteins, and its modulation in NaCl tolerance makes it an interesting target for manipulation in plants. It contains sequence information for adjacent Cys-4 and His/Cys-3 zinc-finger domains that appear to bind adjacent G-rich triplet motifs in DNA (Bastola et al., 1998). It also contains an acidic region characteristic of DNA-binding proteins that interact with other proteins (Kakidani and Ptashne, 1988) and therefore is likely to function as a transcription factor in plants. *Alfin1* is expressed predominantly in roots, appears to be unique or a low-copy gene in the alfalfa genome, and shows conservation among such diverse plants as alfalfa, rice, and Arabidopsis (Winicov and Bastola, 1997). These characteristics, in addition to in vitro binding to promoter fragments of the root-specific *MsPRP2* gene that is also NaCl inducible (Winicov and

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Abbreviations: CaMV, cauliflower mosaic virus; MCS, multiple cloning site; SH, Schenk and Hildebrandt.

Deutch, 1994; Deutch and Winicov, 1995), suggested that it may have a significant function in plant-root gene expression and contribute to gene regulation in NaCl tolerance.

To test the functions of Alfin1, we made constructs of the *Alfin1* cDNA in the sense and antisense orientations, driven by the strong CaMV 35S promoter, transformed alfalfa, and looked for *MsPRP2* expression as a potential reporter for Alfin1 activity in vivo. The antisense transformants demonstrated that normal *Alfin1* transcript levels were essential for plant development in soil. However, antisense transformation only minimally affected callus growth on control medium. Nonetheless, increased or decreased *Alfin1* expression in the transformed callus correlated positively with relative growth in NaCl-containing medium in culture. In addition, we were able to monitor the mRNA levels of the endogenous alfalfa *MsPRP2* gene. In this paper we report that *Alfin1* overexpression in transgenic plants led to *MsPRP2* accumulation in callus and roots, suggesting that Alfin1 acts as a transcriptional regulator in plants and plays an important role in *MsPRP2* expression in alfalfa. Because transgenic plants overexpressing *Alfin1* also showed improved NaCl tolerance, comparable to our NaCl-tolerant plant previously regenerated from cell culture, *Alfin1* expression must play an important regulatory role that can provide enhanced NaCl tolerance in alfalfa.

MATERIALS AND METHODS

Plant Material

Alfalfa (*Medicago sativa* L. cv Regen S) cell lines were maintained on SH growth medium (Schenk and Hildebrandt, 1972) in continuous light with and without 171 mM NaCl, as described previously (Winicov et al., 1989; Winicov and Button, 1991). Because of the autotetraploid genotype of alfalfa, all experiments were performed with the parent control plant labeled no. 1, which represents the NaCl-sensitive wild type. All transformations were done with material from this plant or the NaCl-tolerant mutant no. 9, originally selected and regenerated from no. 1 (Winicov, 1991). The NaCl-sensitive parent and NaCl-tolerant plants regenerated from the NaCl-tolerant cell cultures (Winicov, 1991) were maintained in the greenhouse and propagated by cuttings. The influence of NaCl on plant growth was determined on replicate rooted cuttings of plants established in Conetainers in perlite and watered daily with one-quarter-strength Hoagland solution (Hoagland and Arnon, 1938), with or without the indicated concentrations of NaCl, as described previously (Winicov, 1991). All plant material was harvested at the same time of day.

Recombinant Plasmid Construction

The full-length coding *Alfin1* clone (pA50) consists of a 904-bp fragment of *Alfin1* cDNA (accession no. L07291) in pBluescript SK− (Stratagene). It contains a 30-bp 5′-untranslated leader, a complete 771-bp coding sequence, and 103 bp of the 3′-untranslated region, including the translation termination codon (Winicov, 1993). This cDNA fragment was cloned in the sense and antisense orientations in the MCS of the binary expression vector pGA643 (An et al., 1988), as shown in Figure 1.

To generate the sense construct, the 939-bp *Hin*dIII-*Xba*I fragment from pBluescript SK− was first subcloned in pFLAG (International Biotechnologies Inc., New Haven, CT), designated as PF-pA50, to gain a restriction site suitable for cloning the cDNA fragment in pGA643. The 957-bp *Hin*dIII-*Bg*II fragment from PF-pA50 containing Alfin1 cDNA was then ligated to pGA643 in the MCS 3′ to the CaMV 35S promoter to give pGA-sense. This clone was predicted to give the complete *Alfin1*-coding transcript, but unlike the endogenous *Alfin1* mRNA it carried additional sequences from the vector in its 3′-untranslated region.

To generate the antisense construct (pGA-ATS), the 944-bp *Cla*I-*Xba*I fragment from pA50 (pBluescript SK−) was ligated directly into the pGA643 MCS. Although another *Cla*I site has been reported upstream of the MCS in pGA643, we found that only the *Cla*I site in the MCS, indicated in Figure 1, was cut by the enzyme.

The plasmids pGA-sense, pGA-ATS (antisense), and pGA643 (vector) were propagated in *Escherichia coli* strain MC1000 (a gift from Dr. G. An, Washington State University, Pullman) in the presence of tetracycline. The freeze-thaw method, as described by An et al. (1988), was used to transform *Agrobacterium tumefaciens* LBA 4404 (Hoekema et al., 1983) with the recombinant binary plasmid. Transformed colonies were selected on 12 mg/L rifampicin and

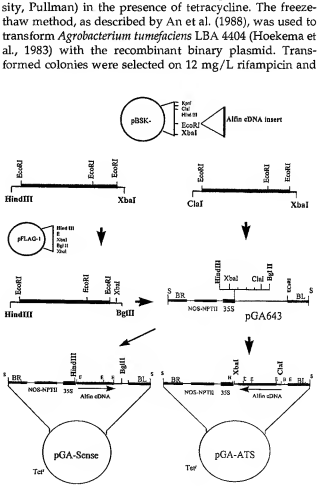

Figure 1. Schematic representation of *Alfin1* sense and antisense constructs used in transformation of alfalfa. Restriction sites are as follows: E, *Eco*RI; H, *Hin*dIII; B, *Bg*lI; and S, *Sal*I. BR and BL are T-DNA right and left borders, respectively (An et al., 1988).

6 mg/L tetracycline. Recombinant transformed colonies were identified by colony hybridization using the *Alfin1* 670-bp *Eco*RI fragment from pA50 (Sambrook et al., 1989).

Plant Transformation

Alfalfa NaCl-sensitive wild-type parent plant no. 1 (Winicov, 1991) leaves were transformed by *A. tumefaciens* cocultivation on SH growth medium, including 2 mg/L 2,4-D and 2 mg/L kinetin (Schenk and Hildebrandt, 1972), and supplemented with 50 μM acetosyringone (Aldrich) for 30 to 60 min at room temperature. One of the successful transformations was carried by cocultivating *A. tumefaciens* carrying the pGA-ATS with immature ovaries from the NaCl-tolerant alfalfa IW9 line (Winicov, 1991). After 2 to 6 d on callus medium the explants were transferred to selection medium (SH medium supplemented with 300 mg/L carbenicillin and 100 mg/L kanamycin) and incubated for 3 to 4 weeks. The resistant calli were subcultured on the selection medium on a monthly basis. Plants were regenerated from the transformed calli on SH medium (without hormones) supplemented with 100 mg/L kanamycin. Plants with well-defined shoots and roots were transferred to peat moss and subsequently to soil.

DNA Extraction and PCR Analysis

Genomic DNA was extracted from 0.5 g of frozen callus or leaves using DNAzol genomic DNA isolation reagent (Molecular Research Center, Inc., Cincinnati, OH), as described by the manufacturer. PCR was carried out in a 25-μL total reaction containing 250 ng of genomic DNA, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1% Triton X-100), 100 μM deoxynucleoside triphosphates, 0.2 μM each of the forward (primer common to all PCR analyses in this section = 5′ CCA CTA ATT CGT CCT GCT GG 3′) and the reverse sequence primers (Midland Certified Reagent Co., Midland, TX) (PS for sense [5′ CCA GTC CCT CTC CTG CAT TC 3′], PA for antisense [5′ GGA CAA GGT GCA ACC TGT GG 3′], and PG for vector [5′ AAG TGT GCT TGA GCT CGG TC 3′]), and 0.25 unit of *Taq* polymerase (Promega). The forward sequence primer was from position 2432 bp and the reverse primer was from 3404 bp for the pGA-vector, 3356 bp for the pGA-sense, and 3359 bp for the pGA-antisense DNA sequence of the T-DNA right border. This combination of PCR primers gave 973-, 926-, and 928-bp products, respectively.

The Gene Amp PCR System (model 2400, Perkin-Elmer) was programmed for an initial denaturing temperature of 94°C for 4 min, a second denaturing temperature of 94°C for 1 min, an annealing temperature of 62°C for 90 s, and an extension temperature of 72°C for 1 min. The reaction was carried out for 35 cycles. An additional extension at 72°C followed for 7 min after completion of the final cycle.

RNA Extraction and Blot Analysis

Total RNA was extracted from roots and shoots containing both leaves and stems from plants grown for 17 d with and without 128 mM NaCl, or callus grown for 1 month with and without 171 mM NaCl, and analyzed under high-stringency hybridization and wash conditions, as described previously (Winicov and Deutch, 1994; Winicov and Krishnan, 1996). Northern analysis for *Alfin1* was done with the 670-bp *Eco*RI large fragment from pA50; for *MsPRP2*, the probe was the *Eco*RI fragment from pA9 (Winicov and Deutch, 1994); the constitutively expressed *Msc27* was probed with the *Pst*I fragment (Gyorgyey et al., 1991); and the 763-bp *Eco*RI-*Bgl*II fragment from pGA643 (the region between the 3′ end of the MCS and the T-DNA left border) was used to detect transgenic *Alfin1* expression. Gel-purified fragment probes were labeled with [^{32}P]dCTP using the random primer-extension system (DuPont-NEN).

RESULTS

Alfalfa Calli Transformed with Sense and Antisense *Alfin1*

NaCl-sensitive alfalfa cells were transformed with pGA-sense, pGA-ATS (antisense), and the vector pGA643. Many kanamycin-resistant lines were isolated from independent transformations in three different experiments. A total of 22 independent transformed lines were obtained with pGA-sense, 14 independent lines were obtained with pGA-ATS, and comparable numbers were obtained using the empty vector pGA643. No consistent differences in cell growth were observed between transformants of the different constructs, although significant growth differences could be seen between independently transformed cell lines. Only transformed calli showing good growth on kanamycin were further maintained and analyzed. Kanamycin-resistant transformants were confirmed by PCR to carry the appropriate inserts (data not shown).

The influence of transformation with *Alfin1* was measured by alfalfa callus growth on SH medium with and without 171 mM NaCl, as shown in Table I. Two NaCl-sensitive cell lines (1,1 and 1,5) were independently initiated in culture. They showed 92% and 84% growth inhibition by NaCl, respectively, as measured by an NaCl-dependent increase in callus wet weight after 4 weeks of growth. The 1,1 cells transformed with pGA-sense showed less growth inhibition by NaCl than those transformed by the pGA643 vector alone. In contrast, 1,5 cells transformed with the pGA-ATS appeared to grow somewhat more slowly on the control medium and were more sensitive to growth inhibition by NaCl than the pGA643 vector-transformed cells. These results were consistent with our hypothesis that Alfin1 helps to maintain cellular functions in our NaCl-tolerant alfalfa. However, none of the sense transformants was able to grow as well on 171 mM NaCl as on the control SH medium.

Overexpression of *Alfin1* in Transgenic Callus Increases *MsPRP2* mRNA Levels

Alfin1 expression was determined in the pGA-sense-transformed callus by northern analysis of total RNA using the constitutively expressed *Msc27* gene probe to monitor RNA concentrations in each lane. In Figure 2, the results show clearly that *Alfin1* expression was greatly enhanced in the S1, S2, S4, and S6 pGA-sense-transformed cell lines compared with untransformed and vector-transformed

Table I. *Cell growth of transformed and untransformed alfalfa cell lines*

Cell Line	Kanamycin	Growth[a]	
		0 NaCl	171 mM NaCl
		g wet wt/plate	
1,1-Untransformed	−	5.49 ± 0.81 (n = 2)	0.90 ± 0.47 (n = 3)
1,1-t-Vector (3)[b]	+	4.34 ± 1.35 (n = 4)	1.08 ± 0.20 (n = 6)
1,1-t-*Alfin1*-sense (6)[b]	+	5.06 ± 1.13 (n = 7)	1.63 ± 0.38 (n = 9)
1,5-Untransformed	−	5.36 ± 0.84 (n = 3)	1.30 ± 0.48 (n = 3)
1,5-t-Vector (2)[b]	+	3.83 ± 0.27 (n = 6)	1.25 ± 0.27 (n = 6)
1,5-t-*Alfin1*-antisense (4)[b]	+	3.39 ± 0.91 (n = 7)	0.93 ± 0.23[c] (n = 6)

[a] Growth (means ± SD) after 4 weeks on SH medium ± 171 mM NaCl, using an initial inoculum of about 0.1 g/callus and five calli/plate. n = number of plates. [b] Number in parentheses, Number of different individual transformants included in test. [c] Brown, dead callus.

cells. Some variability in the levels of expression was observed between different transformants, consistent with the prevalent variability resulting from independent transformation events. Concurrent with the enhanced *Alfin1* expression in the transgenic cells we also found significantly increased levels of *MsPRP2* transcripts. The levels of *MsPRP2* transcripts found in pGA-sense-transformed cells were higher than those found in NaCl-tolerant cells grown in the presence of NaCl, and we could not detect further NaCl-induced enhancement of the high levels of *MsPRP2* mRNA accumulation in the pGA-sense-transformed callus. Because recombinant Alfin1 was shown to bind to promoter fragments of *MsPRP2* in vitro (Bastola et al., 1998), the enhanced levels of endogenous *MsPRP2* transcripts in callus overexpressing *Alfin1* suggest that Alfin1 regulates alfalfa *MsPRP2* expression in vivo.

Phenotype of *Alfin1* Sense and Antisense Transgenic Plants

To investigate the molecular and growth characteristics influenced by *Alfin1* numerous plants were regenerated from pGA-sense-transformed calli and calli transformed with the vector alone. Three pGA-sense-transformed plants, regenerated from independent transformations events, were maintained for molecular and growth studies. All three plants grew well, flowered, and set seed. The sense transformants appeared normal, although young leaves were somewhat broader than those from the parent plant and appeared to senesce somewhat earlier.

Calli transformed with the pGA-ATS construct regenerated shoots readily, but root development was poor. Treatment of the regenerating shoots with 5 μM naphthalene acetic acid gave some root development, but none of the dozen plantlets transferred to soil survived for more than 2 weeks. Only one pGA-ATS-transformed plant survived in soil for about 6 months, but it remained severely dwarfed in both root and shoot growth. These results strongly indicated that *Alfin1* antisense expression was deleterious to growth and root formation and that *Alfin1* transcripts were necessary for plant development in soil, although antisense did not have a similar impact on callus growth in normal SH medium.

Overexpression of *Alfin1* in Transgenic Plants Increases *MsPRP2* mRNA Levels in Roots

Three of the primary transformed plants with pGA-sense constructs were analyzed for tissue-specific expression of the *Alfin1* transgene and its putative target gene *MsPRP2*. Gel-blot analysis of leaf total RNA from soil-grown plants shown in Figure 3 confirmed that the pGA-sense-transformed plants showed high levels of *Alfin1* mRNA expressed from *Alfin1* under the control of the CaMV 35S promoter, in contrast to the untransformed parent plant. The presence of the transgene transcripts was demonstrated by probing of the same blot with the *Bgl*II/*Eco*RI fragment of the pGA643 vector, which is adjacent to the 3′ end of *Alfin1* cDNA and is apparently transcribed in *Alfin1* sense mRNA in the transformants.

Figure 4 shows similar results from the *Alfin1*-overexpressing transgenic plants grown in one-quarter-strength Hoagland solution. The *MsPRP2* transcript levels increased in the roots of the *Alfin1*-overexpressing plants (Fig. 4). The vector-transformed plant no. 1 showed somewhat increased levels of *MsPRP2* mRNA in roots, but this level was not maintained in the presence of NaCl. In fact, the *MsPRP2* mRNA levels were comparable from NaCl-grown control no. 1 and the vector-transformed no. 1 plants. In contrast, the three transgenic plants overexpressing *Alfin1* maintained proportionately higher levels of *MsPRP2* mRNA in roots after growing for 17 d on 128 mM

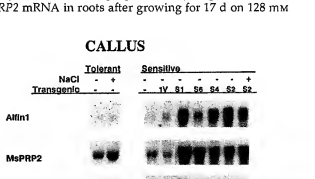

Figure 2. Northern analysis of *Alfin1* and *MsPRP2* expression in control and transgenic calli from *Alfin1* sense transformants. Lanes 1 and 2, RNA isolated from untransformed NaCl-tolerant callus grown with or without 171 mM NaCl for 4 weeks; lane 3, RNA isolated from untransformed NaCl-sensitive callus; lane 4, RNA isolated from NaCl-sensitive callus transformed with pGA vector (1V); lanes 5 to 9, RNA isolated from NaCl-sensitive callus transformed with *Alfin1* sense construct (S1, S2, S4, and S6 are independently transformed lines); and lane 9, RNA isolated from S2-transformed callus grown in 171 mM NaCl. Each lane contained 10 μg of total RNA. Each blot was hybridized sequentially with the following probes: *Alfin1*, the large *Eco*RI fragment (Fig. 1); *MsPRP2*, the carboxy-terminal and 3′-untranslated region fragment (Winicov and Deutch, 1994); and *Msc27*, the fragment of a constitutively expressed alfalfa gene.

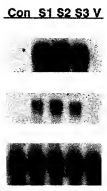

Figure 3. Northern analysis of *Alfin1* expression in control and transgenic plants from *Alfin1* sense transformants. RNA was isolated from leaves of control and transgenic plants. Lane Con, No. 1 control NaCl-sensitive parent plant for all transformations; lanes S1, S2, and S3, plants transformed with the *Alfin1* sense construct and regenerated from transformed callus; and lane V, vector-transformed plant. Each blot was hybridized sequentially with the following probes: *Alfin1*, large *EcoRI* fragment (Fig. 1); pGA-vector, *EcoRV/BglII* fragment from pGA643 to show readthrough of the *Alfin1* transgene; and *Msc27*, fragment of a constitutively expressed alfalfa gene. Each lane contained 10 μg of total RNA.

NaCl-supplemented one-quarter-strength Hoagland solution. The mRNA profiles from NaCl-tolerant no. 9 plants are shown for a comparison. Whereas high levels of *Alfin1* mRNA were found in both roots and leaves because of the 35S promoter control of the transgene, *Alfin1* overexpression had a negligible effect on *MsPRP2* transcript levels in leaves of transgenic plants grown on one-quarter-strength Hoagland solution. NaCl treatment did not further enhance the *MsPRP2* mRNA levels in the transgenic plants, as shown in Figure 4. These results support the Alfin1 functional role in *MsPRP2* expression primarily in roots and indicate that additional tissue-specific factors contribute to the differences observed in *MsPRP2* mRNA levels between roots and leaves.

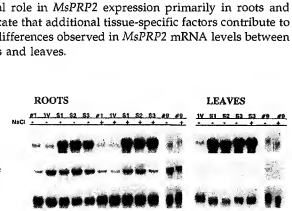

Figure 4. Northern analysis of *Alfin1* and *MsPRP2* expression in control and transgenic plants from *Alfin1* sense transformants grown in one-quarter-strength Hoagland solution with or without 128 mM NaCl. RNA was isolated from roots and leaves of control plants and plants tested for NaCl tolerance described in Table II legend. Lanes #1, Parent wild-type control; lanes 1V, control transformed with empty vector; lanes #9, NaCl-tolerant plant regenerated from NaCl-tolerant callus; and lanes S1, S2, and S3, parent no. 1 transformed with pGA-sense. The blot was hybridized sequentially with probes as described for Figure 2. Each lane contained 10 μg of total RNA.

Effect of *Alfin1* Overexpression on the NaCl-Tolerance Characteristics of the Transgenic Plants

To determine if *Alfin1* overexpression had an effect on the NaCl-tolerance phenotype of the transgenic plants, we compared the growth characteristics of the three pGA-sense-expressing transgenic plants with those of the wild-type NaCl-sensitive parent plant (no. 1), vector-transformed plants, and our previously regenerated NaCl-tolerant plant IW9 (Winicov, 1991). Tolerance was measured as relative new growth obtained from established transgenic and control plants that had been cut back to the crown and then treated for 17 d with 128 mM NaCl. As shown in Table II, one parental control and one vector-transformed plant died from the NaCl treatment. All pGA-sense-expressing transgenic plants and our NaCl-tolerant IW9 plants survived and grew two to three times as well as the parent and vector-transformed controls. It is important to note that IW9 had maintained its significant NaCl-tolerant characteristics for more than 9 years after propagation by cuttings in the greenhouse. All three independently regenerated transgenic plants overexpressing *Alfin1* showed growth characteristics similar to or better than those of our NaCl-tolerant IW9. Vector-transformed controls were as NaCl sensitive as the parent plant. These results are consistent with data from another experiment, which tested tolerance to 171 mM NaCl in plants established for only 1 week. That experiment showed 14%, 43%, 57%, and 86% survival of no. 1 (parent), sense-1, sense-2, and IW9 plants, respectively, after 9 d of NaCl treatment. The results from both experiments indicate that *Alfin1* overexpression can provide increased NaCl tolerance in alfalfa.

Alfin1 and *MsPRP2* steady-state mRNA levels were determined for the NaCl-treated and control plants at the time of the harvest described in Table II and are shown in Figure 4. The S1, S2, and S3 pGA-sense transgenic plants had high levels of *Alfin1* and *MsPRP2* mRNA in roots, but not in shoots, regardless of growth in 128 mM NaCl, although some NaCl-dependent decrease in *MsPRP2* mRNA

Table II. *Growth properties of Alfin1-sense-transformed plants on 128 mM NaCl*

Multiple rooted cuttings from each plant were established in individual Conetainers in perlite for 6 weeks and grown on one-quarter-strength Hoagland solution. All shoots were then cut back to the crown. Growth was continued from that point on one-quarter-strength Hoagland solution supplemented with 128 mM (0.75%) NaCl. The newly regrown shoots were harvested and weighed after 17 d. Data are means ± SD.

Plant	Survival	New Leaf Growth	Percentage
		g/plant	
No. 1 (parent)	4/5	0.56 ± 0.32	100
No. 1 + vector	3/4	0.42 ± 0.32	75
No. 1 + sense-1[a]	7/7	1.40 ± 0.17	250
No. 1 + sense-2[a]	7/7	1.85 ± 0.23	330
No. 1 + sense-3[a]	3/3	1.45 ± 0.32	259
IW9[b]	7/7	1.10 ± 0.18	196

[a] Plants sense-1, sense-2, and sense-3 correspond to S1, S2, and S3 shown in Figure 4. [b] NaCl-tolerant plant regenerated after selection in tissue culture from parent plant no. 1 (Winicov, 1991).

levels is apparent in the S3 transgenic plant. The *MsPRP2* transcript levels appear to be higher in the pGA-sense transgenic plants than in our NaCl-tolerant plant no. 9. Although Table II shows significant differences in the NaCl tolerance of the plants at 128 mM NaCl after 17 d, we did not detect comparable levels of NaCl inducibility of *MsPRP2* mRNA accumulation (Fig. 4), as had been seen in plants treated with 171 mM NaCl for 7 d (Winicov and Deutch, 1994). Whether this difference was due to the lower NaCl concentration or to plant adjustment after a longer time of growth in NaCl will have to be determined and correlated with levels of MsPRP2 protein accumulation when plants are grown for prolonged periods in NaCl.

DISCUSSION

Overexpression of *Alfin1* was engineered in transgenic callus and alfalfa plants under the control of the strong CaMV 35S promoter. Our previous experiments suggested that Alfin1 was likely to function as a transcription factor, since we had shown sequence-specific DNA binding of the recombinant protein in vitro and specific binding to promoter fragments of the *MsPRP2* gene from alfalfa (Bastola et al., 1998). In this paper we are able to show in callus and plants overexpressing *Alfin1* a concomitant increase in the endogenous *MsPRP2* mRNA levels, indicating that the *Alfin1* gene product regulates *MsPRP2* expression in vivo from its normal promoter. These results are consistent with our prediction that Alfin1 is a transcription factor, regulating plant gene expression, and acts in a dominant fashion in overexpressing transgenic plants.

Although *Alfin1* was expressed from the 35S promoter in both roots and leaves, significant *MsPRP2* transcript induction from its natural promoter in the transgenic plants was detected in callus and roots, the tissues in which *Alfin1* is primarily expressed (Bastola et al., 1998). Small differences in *MsPRP2* mRNA induction by *Alfin1* overexpression were observed in leaves of soil-grown plants (data not shown) but not in plants grown on one-quarter-strength Hoagland solution, suggesting subtle variation due to the nutritional state of the plants. The differential response in leaves and roots to high levels of *Alfin1* mRNA could result from the presence of a transcriptional or posttranscriptional inhibitor of *MsPRP2* transcript accumulation in leaves or may indicate the requirement for additional root-specific transcription factors for high levels of expression from the *MsPRP2* promoter. Additional experiments should differentiate between these two possibilities. The callus complement of participating factors in *MsPRP2* expression appears similar to that of the root, because *Alfin1* overexpression led to a significant increase of *MsPRP2* transcripts in callus culture.

The plant phenotype of pGA-ATS transformants was striking in its inability to sustain growth in soil, especially since we observed no substantially altered phenotype in antisense-expressed callus grown on SH medium. These results suggested a low level of redundancy for Alfin1 function and demonstrated that maintenance of *Alfin1* expression was essential for root development and plant growth in soil. Another function affected by *Alfin1* antisense expression could be root-shoot communication via the vascular system, which suggests that the Alfin1 protein may regulate other genes in addition to *MsPRP2*. On the other hand, overexpression of *Alfin1* showed no major visible phenotype, even though it was inappropriately expressed in the shoot.

Because *Alfin1* was first cloned from NaCl-tolerant alfalfa callus (Winicov, 1993), our demonstration of improved NaCl tolerance in the transgenic plants overexpressing *Alfin1* significantly associates the product of this gene with improved NaCl tolerance. However, its relationship to the mutation(s) that allowed the regeneration of our NaCl-tolerant plants, such as IW9 (Winicov, 1991), remains unclear. Transgenic plants have been engineered in a number of laboratories to overexpress single genes, which are known to be up-regulated by NaCl/drought stress in prokaryotes or plants with incremental improvements in NaCl tolerance (Tarcyznski et al., 1993; Kishor et al., 1995; Pilon-Smits et al., 1995; Xu et al., 1996). However, NaCl tolerance has also been considered to be a quantitative trait (Foolad and Jones, 1993), and the molecular mechanisms by which

Table III. *Alfin1-binding sites found in NaCl/drought stress-induced promoter sequences*

Selection of potential *Alfin1*-binding sites was made for the coding strand on the basis of at least two adjacent triplets, one of which is GTG and the other of which is bordered by a G as defined by in vitro *Alfin1* binding (Bastola et al., 1998). Additional sites (not shown) were found on the noncoding strand in many of these gene promoters. Numbers in parentheses indicate accession numbers.

Gene and Plant	Sequence	Ref.
MsPRP2, alfalfa	−299 5′ GTGGGG 3′ −289	Bastola et al. (1998) (AFO28841)
HVA1, barley ABA response element 2	−93 5′ GTGGCG 3′ −87	Straub et al. (1994) (X78205)
Atmyb2, Arabidopsis	−559 5′ GAAGTG 3′ −555	Urao et al. (1993) (D14712)
	−461 5′ GTGTGG 3′ −435	
	−222 5′ GCCGTG 3′ −217	
rab28, maize	−378 5′ GTCGTGCAG 3′ −360	Pla et al. (1991) (X59138)
salT, rice	−1451 5′ GTGCAG 3′ −1446	Claes et al. (1990) (Z25811)
	−843 5′ GTGACG 3′ −828	
Osmotin, tobacco	−1447 5′ GTGGTG 3′ −1442	Ragothama et al. (1993) (S68111)
	−596 5′ GTGGTG 3′ −591	
	−471 5′ GTGGAG 3′ −466	
CDeT27-45, resurrection plant	−703 5′ GTGTGGGCG 3′ −695	Michel et al. (1993) (X69883)

[a] All sequences identified are relative to the first ATG codon.

plants could acquire improved long-term NaCl tolerance, involving the regulation of many genes, are still not understood (for review, see Winicov and Bastola, 1997; Winicov, 1998). Therefore, the possible function of transcription factors associated with stress responses has been of significant interest.

It has been shown that both myc and myb proteins function as transcriptional activators in the *rd22* gene, which is induced by ABA and dehydration (Abe et al., 1997). Many of the NaCl- and drought-induced genes are also induced by ABA, and ABA response element-binding proteins have been cloned (Guiltinan et al., 1990). Other genes responding to NaCl/drought stress and cold are induced in an ABA-independent manner involving the *cis*-acting DRE (DNA regulatory element) (Yamaguchi-Shinozaki and Shinozaki, 1994). Recently, the CBF1 protein (Stockinger et al., 1997), which has been shown to recognize the DRE, was shown to function in enhancing freezing tolerance (Jaglo-Ottosen et al., 1998) in Arabidopsis. These findings suggest that the phenotypic changes involving altered gene expression and resistance to stress might be manipulated through the relevant transcription factors.

Transgenic manipulation of *Alfin1* expression, therefore, is of interest because we have demonstrated *Alfin1* to be a regulatory gene that can influence the expression of *MsPRP2* in a specific manner. An interesting result of the enhanced *Alfin1* expression in our transgenic plants was the finding that these plants demonstrated enhanced NaCl tolerance. It is likely that, as a transcriptional regulator, Alfin1 also influenced the regulation of other genes in our transformed plants, which could have contributed to the enhanced NaCl tolerance observed in our transgenic plants. Table III shows that many of the genes that have been shown to be up-regulated by NaCl/drought stress also contain Alfin1-binding motifs in their promoters. At present, we do not know if any of these other genes are differentially regulated in our *Alfin1*-overexpressing plants, but we might expect to see changes in their expression if *Alfin1* had a general regulatory role in NaCl tolerance.

Future experiments will determine the extent and specificity of plant gene regulation by Alfin1 and the extent to which enhanced *Alfin1* expression could be useful in manipulating plant growth tolerance of environmental conditions.

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This Week's Citation Classic®

von Heijne G. Patterns of amino acids near signal-sequence cleavage sites.
Eur. J. Biochem. 133:17-21, 1983.
[Research Group for Theoretical Biophysics, Department of Theoretical Physics, Royal Institute of Technology, Stockholm, Sweden]

This paper reported a statistical study of the amino-acid sequences of secretory signal peptides. In particular, it demonstrated that only small, uncharged residues are allowed in positions –3 and –1 relative to the site of cleavage between the signal peptide and the mature protein. This observation served as a basis for a scheme that predicts the most likely cleavage site when only the primary sequence of the precursor protein is known. [The *SCI*® indicates that this paper has been cited in over 380 publications.]

Prediction of Cleavage Sites in Secretory Proteins

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September 23, 1988

As a graduate student in Clas Blomberg's Research Group for Theoretical Biophysics at the Royal Institute of Technology in Stockholm, I had one scientifically very fruitful idea: to brush up my rusty high-school French. A demanding teacher made me subscribe to *La Recherche*, a French popular-science magazine. Flipping through its pages one day, I stumbled across a short piece on protein secretion. It described the classic G. Blobel and B. Dobberstein paper[1] that presented the first full-blown version of the signal hypothesis. A small figure illustrated the main idea: a signal peptide initiating cotranslational protein translocation across the membrane of the endoplasmic reticulum (ER). The hydrophobic signal peptide was shown as somehow squeezing through a likewise hydrophobic membrane, ending up, after cleavage, as a freely soluble peptide in the lumen of the ER.

This didn't make sense to me: a hydrophobic peptide ought to become anchored in the membrane, most likely with its charged amino-terminal end remaining in the cytoplasm. I later found out that this was the essence of the so-called "loop model."[2] Fortunately, I didn't know this at the time, or I would never have been drawn into the field of protein sorting.[3] At any rate, this inspired me to write a paper dealing with the energetics of a polypeptide chain passing through a lipid bilayer.[4]

I then got interested in the primary sequences of secreted proteins, and a study of the then-known signal peptides was a fairly obvious step. Again, I didn't know that this had been done before on smaller collections of sequences,[5] and it turned out that my sample was just the right size for discerning what has later become known as the (–3,–1)-rule for the cleavage site between the signal peptide and the mature protein: only small, uncharged residues are allowed in positions –3 and –1. As it happened, an equally well-cited paper[6] with essentially the same message was published by D. Perlman and H. Halvorson within a few weeks of my paper.

The main reason for the many citations is that genes and cDNAs for secretory proteins represent a large proportion of current DNA-sequencing efforts. The (–3,–1)-rule allows one to make a reasonable prediction of the site of signal peptide cleavage in such proteins. If a few thousand new protein sequences are deduced from their DNA sequences per year, and if, say, 20 percent of these represent secretory proteins, and if a good number of the papers reporting these sequences cite the (–3,–1)-rule, one is bound to end up with quite a few centimeters of *Science Citation Index*® column-space. It is thus simple mass-market effects, rather than profound insight or theoretical sophistication, that marks the success of this *Citation Classic*. As for a moral, I guess that the story underlines the well-documented importance of ignorance and French in all scientific work.

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Exhibit 2